Title: Fibroblasts and Immune Cells: At the Crossroad of Organ Inflammation and Fibrosis

Abbreviated Title for Running Head: Fibroblast-immune cell communication

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Abstract

The immune and fibrotic responses have evolved to work in tandem to respond to pathogen clearance and promote tissue repair. However, excessive immune and fibrotic responses lead to chronic inflammation and fibrosis, respectively, both of which are key pathological drivers of organ pathophysiology. Fibroblasts and immune cells are central to these responses, and evidence of these two cell types communicating through soluble mediators or adopting functions from each other through direct contact is constantly emerging. Here, we review complex junctions of fibroblast-immune cell crosstalk, such as immune cell modulation of fibroblast physiology and fibroblast acquisition of immune cell-like functions, as well as how these systems of communication contribute to organ pathophysiology. We review the concept of antigen presentation by fibroblasts among different organs with different regenerative capacities, and then focus on the inflammation-fibrosis axis in the heart in the complex syndrome of heart failure. We discuss the need to develop anti-inflammatory and anti-fibrotic therapies, so far unsuccessful to date, that target novel mechanisms that sit at the crossroads of the fibrotic and immune responses.

Introduction

Organ fibrosis is defined as the excessive deposition of extracellular matrix (ECM) proteins, a process which is advantageous in the short term. In cases of injury, fibrotic remodeling can act to promote tissue repair, as well as provide necessary structure to the damaged organ. Additionally, fibrotic remodeling helps replace dead or infected cells. This process of replacement fibrosis often results in a fibrotic scar that repairs the site of injury. In contrast, fibrosis also occurs in the absence of cell loss due to alterations in the ECM, in what has been termed reactive fibrosis. In both cases, fibrosis leads to impaired organ physiology in the long term, and particularly in the heart, an organ with limited regenerative capacity, results in longstanding pathology and dysfunction contributing to the deadly syndrome of heart failure (HF). Immune cells are often found in fibrotic areas and can secrete pro-fibrotic cytokines that alter the activity of fibroblasts, the major cells producing collagen and other components of the ECM, which adopt a profibrotic function in response to this interaction. Conversely, fibroblasts express immune pattern recognition receptors (PRR), cytokine and chemokine receptors that allow them to adopt "immune like" functions when engaged. These include the secretion of pro-inflammatory cytokines and chemokines which attract immune cells to the fibrotic site as part of the immune response as well as the expression of molecules involved in antigen presentation.

Here, we review recent discoveries of molecular players in immune cell-fibroblast crosstalk. We comment on different cellular states of immune cells and fibroblasts adopting functions from each other during homeostasis and throughout the course of disease. We present recently discovered novel functions of fibroblasts as antigen presenting cells in the joint, gut, tumors, and heart. Additionally, we focus on fibroblast states and immune cell subsets that work in a network

central to cardiac remodeling and have consequences in the different etiologies of the complex syndrome of HF. This review provides an opportunity to explore what may be therapeutically targeted at the interphase of fibroblasts and immune cell physiology to control organ inflammation and fibrosis.

Immune cell contribution to tissue and organ fibrosis

Fibroblasts and Immune cells in a nutshell

Fibroblasts are responsible for providing organ rigidity and flexibility, an effect achieved by the deposition of ECM proteins. They are highly plastic stromal cells that adopt different phenotypically-distinct states in response to extracellular cues in their microenvironment. Transition between states often occurs in disease settings with defined temporal stages that align with immune cell infiltration. Although at least four fibroblast states have been identified in multiple organs, different insults and changes in the organ microenvironment can lead to more complex intermediate states, such as in response to myocardial ischemia, as previously reviewed (1, 2) (Figure 1A).

In addition to ECM synthesis and growth factor secretion, fibroblasts can release chemokines and cytokines, effectively adopting immune-like functions (Figure 1B), a process that has been reported in multiple organs, as well as in tumor associated fibroblasts (3). Fibroblasts have also received recent attention as communication hubs within organs, showing a surprising breadth of interaction with other resident cells including epithelial cells, endothelial cells, cardiomyocytes, tumor cells and immune cells (4, 5). While the production of ECM, cytokines and chemokines is common to all fibroblasts (6, 7), it is becoming evident that the heterogenous fibroblast origins and the high degree of fibroblast phenotypic plasticity can be imprinted by the tissue niche as an adaptive mechanism that helps with the mechanical and physiological needs of each organ to

maintain homeostasis and respond to insults (8). These states are shaped by resident and infiltrating immune cells, which contribute to fibroblast cell plasticity and organ adaptation to stress.

Immune cells are generally categorized in the two arms of immunity: innate and adaptive. Ongoing characterization of different immune cell phenotypic states has revealed that this categorization may not be so stringent, with some immune cell types sharing properties of both arms (9, 10). Broadly, innate immune cells include neutrophils, tissue resident and tissue-infiltrating macrophages, and dendritic cells (DC), which present antigens to lymphocytes and initiate adaptive immune responses and serve as the necessary link between innate and adaptive immunity. Macrophages constitute one of the most plastic immune cell types in the body, and they participate in phagocytosis of debris and secretion of cytokines that have effects on immune and organ stromal cells, including fibroblasts. A current simplistic designation of macrophages is based on the surface markers C-C motif Chemokine Receptor 2 (CCR2) and CX3C motif chemokine receptor 1 (CX3CR1), which define their bone marrow or yolk sac origin and whether they are organ infiltrating as part of the immune response, or tissue resident, respectively (11, 12). It is now clear that resident macrophages transmit signals and modify tissue properties, providing central homeostatic regulation of organ physiology. For example, monocyte-derived osteoclasts in the bone play a key role in remodeling in the context of mechanical stress (13); in skeletal muscle, resident macrophages orchestrate muscle regeneration in response to injury cooperating with multiple immune cells (14); in the brain, they participate in synaptic pruning and contribute to vascular and neural development by acting as endothelial cell chaperones (15); and in the heart, tissue-resident macrophages facilitate electrical conduction and synchronize cardiac rhythm (16).

Adaptive immune cells comprised of B and T lymphocytes (B and T cells) are uniquely characterized by having antigen specificity and immune memory. Upon antigen recognition by the B cell and T cell receptors (BCR and TCR), they undergo clonal expansion and activation and release antibodies (plasma B cells), cytokines and cytotoxic granules (T helper $CD4^+$ and T cytotoxic $CD8^+$ T cells, respectively) and differentiate into specialized subsets with distinctly unique and durable immune functions, which has been extensively reviewed (17, 18). In brief, cytotoxic $CD8^+$ T cells recognize antigens presented by DC on major histocompatibility complex I (MHC-I), and helper $CD4^+$ T cells do so through DC MHC-II, both of which can also be expressed by fibroblasts and participate in immune cell-fibroblast cross-communication, as reviewed here. T cells and B cells mostly reside in the lymphoid organs and the gut and circulate throughout the body. However, recent evidence has revealed B cell and T cell pools that are transcriptionally different than the circulating pools residing in most organs, and possibly influenced by the organ stroma (19-21). A logistic regression model of human organs single cell sequencing data showed that while resident adaptive immune cells are transcriptionally similar in the human heart, kidney and skeletal muscle, resident innate immune cells are highly tissue adaptive and less similar amongst different organs (4). Thus, immune cells and fibroblasts can influence each other, and innate immune cells are more shaped by specific organ niches than adaptive immune cells, yet all participate in immune and fibrotic responses through several mechanisms of action reviewed herein.

Profibrotic functions of immune cells through soluble factor secretion in homeostasis and disease.

Innate and adaptive immune cells are sources of cytokines, some of which have pro-fibrotic actions by signaling through their receptors expressed on fibroblasts. Under homeostatic

conditions, macrophages communicate with fibroblasts through the release of Platelet Derived Growth Factors (PDGFs) acting on Platelet Derived Growth Factor Receptor (PDGFR) expressed on fibroblasts (22). This communication results in the expression of pro-survival genes, and has been demonstrated to act as a mechanism of homeostatic maintenance by resident macrophages at steady-state in multiple organs. In the heart, this axis is central to fibroblast survival, as *Pgdfrα-/-* mice have 50% less fibroblasts in the heart than control littermates (23).

In the aftermath of heighted inflammation, profibrotic cytokines such as IL-13, IL-4, and IL-5 are released by organ infiltrated T cell subsets ($CD4^+$ T helper type 2 cells-Th2), macrophages, and other innate immune cells (24). These cytokines are canonical drivers of type 2 immunity and serve to regulate tissue repair and fibrosis following injury in primarily mucosal tissues such as the intestines and the lung (25). IL-33 produced by epithelial cells and fibroblasts is often the initiator of such fibrotic responses in the lung, intestine, the kidney, and heart (26). Preclinical acute kidney injury (AKI) studies reveal IL-33 regulates fibrosis in the kidney and the heart through direct binding of IL-33 to cardiomyocytes, establishing IL-33 as a critical component of the kidney-heart axis in AKI-induced cardiomyopathy (27). IL-4, IL-5 and IL-13 have been implicated as a key drivers of fibrosis in the skin, the adipose tissue, and the lung in pre-clinical models of inflammation and fibrosis (28-31). Besides these, cytokines not thought to be pro-fibrotic in nature, such as IL-17A and IFN-γ, have also been recently described to have pro-fibrotic actions on fibroblasts: IL-17A, produced by CD4+ T helper type 17 cells (Th17 cells) was shown to activate synovial fibroblasts which then increased production of GM-CSF, necessary for the development of rheumatoid arthritis in mice (32). Additionally, IFN-γ released by T helper type 1 cells (Th1) is necessary for cardiac fibrosis in experimental models of heart failure and in cardiac repair, although protective against fibrosis in experimental autoimmune

myocarditis and chronic viral myocarditis (33-36). Similarly, Type I Interferons have also recently been reported to act on fibroblasts in the onset of cardiac fibrosis in human hearts with congenital heart block (37, 38).

Perhaps the most widely recognized pro-fibrotic cytokine is transforming growth factor β (TGFβ), which is synthesized and released by activated fibroblasts. TGFβ is also released- although to a lesser extent- by innate and adaptive immune cells. The reported production of TGFβ by $CCR2^+$ macrophages and $CD4^+$ T regulatory cells after phagocytosing apoptotic cells and antigen engagement respectively may also contribute to fibroblast activation and organ fibrosis (39). In the heart, infiltrating macrophage production of IL-12α was deemed to be pro-fibrotic, as IL-12α deficient mice had decreased fibrotic scarring in experimental models of myocardial infarction, and in response to cardiac pressure overload (40). The absence of IL-12α also resulted in a significant decrease in TAC-induced $CD8^+$ T-cell activation in the lung (41). Given that macrophages and fibroblasts are producers of IL-12α, this finding may suggest that blocking their communication can lead to protection against cardiac and lung inflammation and fibrosis. Lastly, the matrix protein osteopontin is highly expressed in atrial infiltrating monocytes in patients with persistent atrial fibrillation, and preclinical studies using combined hypertension, obesity, and mitral valve regurgitation (HOMER) mice identified macrophage-derived osteopontin as a critical driver of fibrosis and inflammatory fibroblast activation in atrial fibrillation (42).

Taken together, immune cell-fibroblast communication through a variety of soluble mediators that include cytokines, growth factors, and matrix metalloproteinases (MMPs), is central to fibroblast function at the steady state and in response to tissue damage, and thus critical for survival (Figure 1 A, B).

In addition, emerging literature demonstrates that immune cells establish direct interactions with fibroblasts and contribute to organ fibrosis. In the lung, in the onset of Idiopathic Pulmonary Fibrosis (IPF), alveolar fibroblast activation is dependent on the proximity with alveolar macrophages. Mechanistic studies showed that only direct co-culture of lung macrophages with lung fibroblasts resulted in pro-fibrotic signals and fibroblast transformation, supporting that macrophages critically regulate pulmonary fibrosis in a spatially and contact dependent manner (43). Additionally, in preclinical pressure overload-induced HF, $CD4^+$ T cells directly adhere to cardiac fibroblasts through α4 integrin-mediated adhesion and promotes fibroblast TGFβ secretion and their transition to profibrotic myofibroblasts in an IFN-γ-dependent manner (33) (Figure 1C-D), additionally supporting that physical interactions between immune cells and fibroblasts modulate tissue fibrosis.

Fibroblast contribution to immune cell responses during homeostasis and inflammation

"Immune like" functions of fibroblasts through soluble factor secretion in homeostasis and disease.

One of the best characterized mechanisms for fibroblast-immune cell cross-communication at steady state is the induction and production of Colony Stimulating Factor 1 (CSF1) with actions on its receptor CSF1R on macrophages, a highly conserved axis with similar function across multiple organs and microenvironments (44). Reticular fibroblast-derived CSF1 regulates splenic red pulp macrophage homeostasis, as shown in studies in which fibroblast deletion of CSF1 drastically diminished the density of red pulp macrophages. Fibroblast-specific deletion of CSF1 did not alter circulating CSF1 abundance, underscoring the importance of soluble factor-mediated fibroblast-macrophage crosstalk in the tissue microenvironment (45). In response to

inflammatory insults, fibroblasts also release several soluble factors that impact resident and tissue infiltrating immune cells and exacerbate the immune response. This is well exemplified in autoimmune diseases and is becoming evident as well in heart disease. Single cell RNA sequencing from patients with Rheumatoid Arthritis (RA), an autoimmune disorder characterized by inflammation of the connective tissue that lines the joint capsule, identified a proinflammatory heparin-binding EGF-like growth factor expressing set of macrophages which was induced by prostaglandin E2 (PGE2) produced synovial fibroblasts. These macrophages then in turn further activated synovial fibroblasts, and in an *in vitro* assay promoted their invasiveness (46). RA patient samples have also revealed the expansion of a subset of fibroblasts located near the perivascular zone of the synovium characterized by increased proinflammatory and chemo-attractive cytokines. Expansion of this subset in patients also correlated to increased leukocyte infiltration of the synovium (47), further supporting the pro-inflammatory role of fibroblasts in the joint during autoimmunity.

In Inflammatory bowel disease (IBD), an autoimmune disease characterized by gut inflammation in part due to an aberrant immune response, fibroblasts express higher levels of the receptor of the IL-6 family cytokine oncostatin M (OSMR) as compared to healthy controls, and these high expressing OSMR fibroblasts sorted from IBD patients showed enhanced expression of proinflammatory cytokines and chemoattractant such as IL-1β, IL-6, CXCL9, and CXCL10 (48). Single cell data from IBD patients also revealed a marked expansion of a subset termed inflammatory associated fibroblasts compared to healthy controls, and the OSM/OSMR axis was identified as one of the most enriched pathways found in this population. Additionally, a comparison between responders and non-responders to anti-TNFα therapy, commonly used in IBD, revealed a strong correlation of non-responders with the enhanced presence of

inflammatory associated fibroblasts (49). Fibroblasts expressing the pro-inflammatory and leukocyte attracting molecules IL-6, IL-11, CXCL2, CXCL5, CCL2, CCL7 have also been identified in IBD patients in a separate study (50), altogether demonstrating that fibroblasts in the gut adopt immune-like functions that contribute to immune cell responses in IBD.

In the inflamed heart, cardiac fibroblasts sense myocardial damage and perpetuate the immune response through pro-inflammatory cytokine secretion and secretion of chemokines to recruit immune cells to sites of damage (Figure 1C) (51). For example, mouse cardiac fibroblasts express CXCL9 and CXCL10, which serve as chemoattractants for T helper 1 cells in the onset experimental heart failure induced by cardiac pressure overload (52). Liu et al. utilized scRNA sequencing to demonstrate dying cardiomyocytes induce TGFβ expression by fibroblasts. Fibroblasts are capable of self-activating via TGFβ induction and have also been shown to activate macrophages to be pro-inflammatory via TGFβ production as well (7, 53). Given that macrophages and fibroblasts are highly responsive to TGFβ and both can produce it during cardiac injury and stress, the failing heart represents a key site of pro-inflammatory crosstalk for macrophages and fibroblasts. In all cases, fibroblasts adopt immune cell like functions that contribute to organ homeostasis as well as inflammation.

Fibroblasts as antigen presenting cells to T cells:

DCs are professional antigen presenting cells (APCs) that efficiently internalize and process antigens displayed by MHC-II molecules on the cell membrane. They also express the co-costimulatory molecules CD80 and CD86 and induce $CD4^+$ T cell responses upon antigen presentation. The fact that MHC-II can be induced on fibroblasts in several organs by proinflammatory cytokines such as IFNγ (54) has led to the appreciation of a novel immune-like

role of fibroblasts as antigen presenting cells at the sites of organ damage, and revealed a new possible target for future therapeutics for autoimmunity, heart disease, and cancer (Figure 2).

In an experimental mouse model of RA, synovial fibroblasts were reported to function as APCs, taking up and processing citrullinated peptides contained by neutrophil extracellular traps (NETs) in the inflamed synovium through a RAGE-TLR9 dependent process. This was regulated by IL17-B and resulted in presentation of the processed peptides on MHC-II molecules and activation of infiltrating $CD4^+$ T cells (55) (Figure 2A). Similarly, in IBD, single cell data from patients showed expansion of a subset of fibroblasts expressing the invariant chain of MHC-II, CD74, critical for MHCII processing and trafficking to the cell surface (56). $CD74^+$ fibroblasts also showed upregulation of CCL19 and CCL21 and the T cell co-stimulatory TNF-superfamily ligand (TNFSF14/LIGHT), further suggesting that gut fibroblasts contribute to T cell immune responses in the gut potentially through antigen presentation (Figure 2B). Perhaps one of the better characterized fibroblasts as APCs are tumor associated fibroblasts (57, 58). In human lung non-small cell carcinoma, as well as in a murine model of lung cancer, MHC-II is induced by IFNy in fibroblasts. Fibroblast specific deletion of MHC-II showed reduction in CD4 T cell expansion within the tumor, decreased survival, and increased tumor growth, whereas MHC-II expressing tumor associated fibroblasts promoted CD4 T cell survival through secretion of the complement protein C1q (57) (Figure 2C).

Cardiac fibroblasts (CFBs) can also express MHC-II and function as APCs in the context of cardiac inflammation induced by transverse aortic constriction (TAC) and by *T. cruzi* infection in mice. Use of the $Nur77^{GFP}$ mouse, a reporter for active antigen engagement of the T cell receptor, demonstrated that cardiac infiltrated $CD4^+$ T cells are restimulated by their cognate antigen by local APCs in the heart (59). CFBs also responded to IFNγ with upregulation of

MHC-II and bared functional antigen uptake, processing, and presentation machinery, reiterating their role as APCs (Figure 2D). *Tcf21*^{iCre}-*Mhcii*$^{fl/fl}$ mice receiving TAC had significantly attenuated adverse cardiac pathology, including less perivascular fibrosis and preserved cardiac systolic function, compared to controls receiving TAC, demonstrating antigen presentation by CFBs to $CD4^+$ T cells is necessary for T cell-mediated adverse cardiac remodeling in the pressure overloaded heart (60). The role of CFB antigen presentation in adverse cardiac remodeling in humans is also supported by scRNA-seq data that shows CFB HLA-DR expression in dilated cardiomyopathy patients that is then reduced after implementation of a left ventricular assist device which lessens the burden of HF(61).

Taken together, fibroblasts are immunologically active in several disease processes both through soluble factor secretion and direct fibroblast-T cell crosstalk through antigen presentation.

Cardiac fibrosis and inflammation: reparative, reactive and involvement in heart failure.

Cellular states of cardiac fibroblasts and the influence of cardiac inflammation

HF is a widespread syndrome characterized by impaired cardiac function, which can arise in response to acute injuries originating in the myocardium, or in response to long-standing systemic stressors that force the heart to adapt to meet metabolic demand. CFBs are a dominant stromal cell in the heart, and are present in both the right and left ventricles, which each have distinct ECM properties and mechanisms of fibrotic remodeling under healthy development and under diseased conditions, as has been elegantly reviewed previously (62). Critically, CFBs have high phenotypic plasticity required to respond to the demands of the heart at steady state, as well as to repair and protect an organ with limited reparative capacity under pathophysiological circumstances. In cases of massive cardiomyocyte death, they adapt to participate in replacement

fibrosis, wherein the accumulation of newly formed ECM takes the place of dying and apoptotic cardiomyocytes. This is a reparative process that allows for continued integrity of the myocardium without which the myocardium would thin and finally rupture. In other cases of cardiac pathology absent of substantial myocardial cell death, accumulation of excessive ECM occurs in what is termed reactive fibrosis. Both types of fibrosis are modulated by the response of CFBs to various neurohormonal, mechanosensitive, and immune mediated factors and contribute to stiffening of the heart, which often leads to arrythmias and defective contractile and relaxation function. As a result, the presence and extent of cardiac fibrosis is associated with a worsening heart failure prognosis (63).

Single cell sequencing has demonstrated regional variation in the transcriptomic expression profiles of fibroblasts based on their relative locations, as well as within the heart, and samples from advanced stage HF patients show expansion of activated CFBs that express well known markers of activation and ECM reorganization (4, 64, 65). Interestingly, comparisons of CFB expression profiles between patients with hypertrophic, dilated, and ischemic cardiomyopathy show remarkably little differences. One explanation for these similarities despite different etiologies could be that these samples are taken from late stage HF patients and fibrotic pathways may converge to a common point in chronic HF. Thus, using preclinical studies to understand the composition of the fibroblast pool and specific mechanisms of immune-mediated fibroblast activation in different HF etiologies is of paramount importance.

In a model of myocardial infarction (MI) in mice, Farbehi et al showed distinct quiescent CFB populations at 3 and 7 days after injury that differed in expression of Sca-1. Activated CFBs then transitioned into myofibroblast sub-populations with pro-fibrotic with upregulated *Tgfb1* and antifibrotic signatures including *Wisp2* and *Sfrp2* which encode proteins with roles in inhibiting

pro-fibrotic WNT signaling (5). Within 4 weeks after MI, matrifibrocytes, a subset of fibroblasts showing increased expression of chondrocyte associated genes, are found in the infarcted area and may play important roles in scar maturation and maintenance(66). Analysis in an angiotensin II (AngII) induced hypertension mouse model of reactive fibrosis showed activated fibroblasts expressing Thbs4 and Cilp as the most fibrogenic cell types, while not expressing α smooth muscle actin (αSMA), a canonical marker of myofibroblast transformation(67). Similarly, in a model of pressure overload induced by TAC in mice, the transcriptional profiles of control and TAC mice at 14 and 28 after surgery were used to identify different clusters of fibroblasts that expand in response to TAC. Additionally, these experiments revealed that the gene *Thbs4* was reported as a key gene expressed in their highest expressing ECM cardiac fibroblast subset as well a subset of interferon responsive fibroblasts (68).

Cardiac fibrosis is also characterized by fibroblast proliferation in addition to ECM production and transformation to myofibroblasts. There is emerging evidence that fibroblasts can enter a proliferation-resistant state, also known as senescent-like state, with reduced ECM secretion, delineating this fibroblast subset from others that exert pro-fibrotic action. These are characterized by the expression of senescence markers such as SA B-Gal, p53 and p16 in experimental models of myocardial infarction and cardiac pressure overload. Genetic ablation of p53 or p16 in mice subjected to cardiac pressure overload showed increased fibrosis, while AAV9 expression of CCN1–an inducer of cellular senescence--led to a 50% reduction in perivascular fibrosis(69), supporting that the cardiac fibroblast senescent like state can prevent excessive fibrosis. However, while this may be beneficial in the short term, the presence of senescent fibroblasts may be detrimental in the long term, a concept deserving of further investigation.

The transition of fibroblasts into different states is regulated temporally through a variety of regulators, including expression of post-transcriptional regulators such as the RNA-binding protein musclebline-like 1 (MBNL1), which induces and stabilizes myofibroblast phenotypes (70). Interestingly, while myofibroblasts are canonically characterized by the expression of alpha smooth muscle actin (encoded by *Acta2*), CFB-specific deletion of *Acta2* did not affect cardiac fibrosis, ejection fraction, or mouse survival post-MI, underscoring how functionally redundant mechanisms are in place in this population of cells (71). However, despite these redundancies, the heterogeneity of the CFB pool, and the plasticity of CFB phenotypes, the effects of specific CFB subsets on reparative vs reactive fibrosis are concrete. For instance, while CFBs are the cardiac cell type in the failing heart expressing the highest levels of family with sequence similarity 114 member A1 (*FAM114A1*), a gene regulating cardiac fibroblast ECM protein expression and TGFβ-dependent migration, CFB-specific deletion of *FAM114A1* ameliorates adverse fibrotic remodeling and impaired contractility post-MI, without inducing cardiac rupture, highlighting the different mechanisms that regulate CFB activity in reparative and reactive fibrosis (72).

Fibrosis in Heart Failure with Reduced Ejection Fraction (HFrEF): Shaping and shifting the fibroblast pool

Heart Failure with Reduced Ejection Fraction (HFrEF) is a category of HF etiologies in which cardiac contractility is impaired. HFrEF can be further subdivided into ischemic and nonischemic HF, reflecting the nature of the insult/injury experienced by the myocardium. In the former, spatial and temporal alterations in fibroblast activity and inflammation propagate reparative fibrosis as they work to scavenge necrotic tissue and deposit a collagenous scar in its place to prevent cardiac rupture. In chronic ischemic HF and nonischemic HFrEF, when the

ventricle experiences hemodynamic stress and stimuli unrelated to CM death, reactive fibrosis occurs. Substantial effort has been made to understand the molecular and cellular mechanisms underlying these different modes of fibrosis, and their subsequent effects on impaired cardiac contractility.

As discussed in this review, fibroblasts and immune cells interface regularly in the heart, as well as in other organs, to perpetuate fibrotic remodeling. In experimental pressure-overload, we have recently demonstrated that tuning $CD4^+$ T cell activation directly modulates cardiac fibrotic remodeling through direct T cell:CFB interactions (73). Furthermore, in addition to their interactions with each other in the myocardium, how these cell types interact with the ECM are of particular importance, as the ECM contains proteins that modulate fibrotic remodeling. For instance, asporin, an inhibitor of TGFβ1, has protective roles in HFrEF, both through inhibiting reactive fibrosis in TAC and preserving cardiomyocyte viability in ischemia reperfusion injury (74). Through recent efforts, key molecular mechanisms underlying interactions between CFBs and the myocardium have emerged. For example, the yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) complex, a transcription factor complex serving as effectors of the Hippo signaling pathway, is a recognized mechanotransduction pathway by which fibroblasts recognize and respond to alterations in the ECM (75). Nuclear localization of YAP occurs in cells following cytoskeletal sensing of mechanical strain, and are inhibited by phosphorylation by Hippo pathway kinases, large tumor suppressor kinase (LATS). Deletion of *Lats* in mice resulted in unbalanced cardiac fibroblast transformation and pervasive myocardial fibrosis, reduced EF, and increased CFB-derived expression of *Ccl7* (76). In primary human cardiac stromal cells, pharmacological targeting of YAP by verteporfin downregulated pathways related to ECM organization, cell migration,

inflammation, and cytokine production as measured by RNA sequencing (77). Deletion of YAP/TAZ in cardiac fibroblasts using the *Col1a2*^{Cre} system in mice undergoing MI resulted in reduced cardiac fibroblast cytokine secretion, resulting in reductions in F4/80^{+} macrophage migration to the infarct and border zone, alongside reduced fibrotic remodeling (78). More recently, YAP deletion in myeloid cells, and targeting myeloid YAP with nanoparticles resulted in decreased fibrosis induced by TAC through mechanisms that involve an anti-fibrotic and pro-resolution phenotype of macrophages deficient in YAP (79). Thus, the cell specificity of the YAP pathway is important in the interphase of the fibrotic and immune responses during reactive fibrosis in mice.

In ischemic HF, the activity by fibroblasts and immune cells is temporally regulated, reflecting the transition from the scavenging of ischemic necrotic tissue to the development of reparative fibrosis. Perpetuating this transition are phases of inflammation, in which infiltrating and resident immune cells play distinct roles in acute and chronic time points post-MI. How these cells affect the remodeling myocardium is highly stage-specific. For instance, CD4^{+} T cells exhibit biphasic cardiac infiltration, and the later infiltrating cells specifically correlate to the progression of chronic HF (80). Similarly, the cardiac fibroblast pool is highly heterogenous, with specific subpopulations playing key roles in different phases post-MI. Single cell analysis of isolated fibroblasts from murine hearts after MI revealed a population of infarct-associated periostin (*postn*$^{+}$) fibroblasts emerging post-MI that play active roles in reparative fibrosis (81). This is not unlike the temporal phases of fibrotic remodeling in skin, another instance of reparative fibrotic remodeling, wherein early and late time points are correlated with pro-inflammatory then pro-fibrotic polarizations of fibroblasts in early then late time points, respectively, of wound healing (82). Following the reparative phase of MI, resolution of inflammation and fibrosis is achieved in

part through the recruitment of anti-inflammatory cells, such as T regulatory cells, which suppress pro-inflammatory cell activity and may directly attenuate fibroblast activity through reducing fibroblast α-SMA expression and secretion of matrix metallopeptidase 3 (83).

Interestingly, while the heart experiences very different stressors in nonischemic HF compared to ischemic HF, there are similarities in how fibroblasts propagate reactive fibrosis in this setting. For instance, TAC also expands a particular subgroup of highly proliferative fibroblasts, which are in this case endocardial-derived, demonstrating a degree of specialization similar to how MI shapes the fibroblast pool (84). In response to TAC, fibroblasts also experience temporally regulated changes in expression of genes associated with different states, stages of activation, and polarity, and ablation of early response proteins such as receptor tyrosine kinase-like orphan receptor 1 and 2 (Ror1/2) resulted in a hyperinflammatory cardiac state (85). Thus, the stages of fibroblast polarization and the times at which they occur have critical functions for inflammation and adverse cardiac remodeling in response to pressure overload. Additionally, as with MI, CFBs are highly influenced by the state of the ECM at the acute and chronic stages of pressure overload. Cardiac accumulation of chondroitin sulfate glycosaminoglycans (CS-GAGs)--proteoglycans secreted by CFBs--has been linked to heightened inflammation and progression of HF. However, abrogated production of CS-GAGs through genetic deletion of chondroitin sulfate N-acetylgalactosaminyltransferase-2 in mice results in worsened cardiac dysfunction 2 weeks after TAC, demonstrating that these factors have protective effects in the acute phase post-TAC, which may differ from their effects in chronic HF (86). This demonstrates that there are similarities in how the CFB pool is shaped in response to ischemic and non-ischemic stimuli, and these similarities may be leveraged to devise therapeutic approaches that may be effective for both categories of etiologies. As an example, salinomycin, a polyether ionophore, was shown to

attenuate pathological fibrotic remodeling in preclinical hypertension induced by angiotensin II and MI, likely by targeting several different pathways in CFBs and cardiomyocytes (87).

In conclusion, the alterations in the myocardium that drive impaired contractility in HFrEF are actively sensed by fibroblasts, and translate into global changes in CFB populations, polarization, and activity at distinct time points of acute and chronic HF. These are tightly regulated by the composition and type of immune response, as fibroblasts and immune cell types communicate in the heart and participate in myocardial repair and maladaptive remodeling (Figure 3, top). While the initiation and progression of ischemic vs nonischemic cardiac remodeling have distinct differences and involve distinct immune responses, there are also similarities in how CFBs respond to these stimuli and crosstalk with immune cells, which may be leveraged in future therapeutic approaches.

Fibrosis in HFpEF: hidden and dispersed.

Heart Failure with Preserved Ejection Fraction (HFpEF) is a complex multi-organ syndrome accounting for roughly 50% of HF diagnoses (88). Therapies that have been successful in treating HF with reduced ejection fraction (HFrEF) patients often fail to do the same for HFpEF patients. For example, the combination of sacubitril/valsartan (angiotensin receptor-neprilysin inhibitor), which was found to lower the rate of hospitalization for HF or death in HFrEF patients, did not affect hospitalization rate or mortality in HFpEF patients (89), despite rodent studies demonstrating the drug treatment ameliorates cardiac fibrosis and cardiac expression of pro-fibrotic genes in preclinical HFpEF(90). As HFpEF often presents in patients with many long-standing comorbidities (such as obesity, hypertension, age, and kidney disease) the HFpEF patient population is highly heterogenous, complicating clinical diagnosis and treatment, as well as accurate/representative preclinical modeling (91). To identify specific mechanisms of HFpEF,

including the progression of cardiac fibrosis and mechanisms of immune-mediated cardiac pathology, several rodent models inducing one or several HFpEF comorbidities are used, and as such, reveal potentially targetable axes in specific HFpEF patient subgroups.

Preclinical studies and improved noninvasive imaging techniques identify cardiac fibrosis in HFpEF, and associate cardiac fibrosis with poor patient outcomes. In patients, cardiovascular magnetic resonance (CMR) is frequently used to measure fibrotic remodeling in patients – myocardial scars and focal fibrosis (often seen in ischemic and non-ischemic HF) are measured by late gadolinium enhancement, while "diffuse" fibrosis, which includes microscopic interstitial fibrosis, is measured by T1 mapping (92). The latter quantifies the myocardial extracellular volume (ECV), which is a surrogate marker of dispersed interstitial fibrosis, and serves as an important predictor of adverse outcomes in HFpEF, demonstrating diffuse fibrosis plays critical roles for HFpEF pathology despite its relatively subtle presentation (93). Endomyocardial biopsy samples from HFpEF patients reveal increases in myocardial collagen I and III gene expression compared with non-HF controls, alongside increases in $CD3^+$ $CD11^+$ and $CD45^+$ leukocytes as measured by immunohistological staining (94). Another study investigating endomyocardial biopsy samples found the prevalence of cardiac amyloidosis to be 14% of their HFpEF patient cohort, when oftentimes cardiac amyloidosis was not suspected (95). These patients experience abnormal amyloid deposition into the myocardium resulting in increased ventricular stiffness, and a study using histological assessment of cardiac fibrosis and amyloid deposition in HFpEF autopsy tissue indicated the presence of this deposition is associated with greater cardiac fibrosis (96). Studies investigating the relationship between amyloid deposition, inflammation, and cardiac fibrosis are necessary to identify mechanisms by which these pathologies may synergize to impair cardiac relaxation. Despite clear evidence of myocardial ECM remodeling in HFpEF,

the extent to which this impairs cardiac relaxation is disputed. The PIROUETTE phase 2 clinical trial testing pirfenidone, an anti-fibrotic agent approved for patients with idiopathic pulmonary fibrosis, successfully reduced myocardial ECV in HFpEF patients (the primary outcome of the study), though left ventricular diastolic function was not impacted, suggesting other factors other than bulk ECM volume contribute to diastolic dysfunction (97), such as cellular changes at the cardiomyocyte level. Additionally, HFpEF endomyocardial biopsies have unique tissue signatures compared to HFrEF right ventricular septal tissue samples, in part characterized by a downregulation in fibrosis-related genes in HFpEF compared to HFrEF as measured by RNA sequencing (98). Thus, the processes governing fibrotic remodeling in HFpEF are likely categorically distinct from those in HFrEF, and may be regarded as simultaneously more subtle and more widespread than the focal fibrotic areas found in HFrEF.

Nevertheless, preclinical models have revealed novel mechanisms of fibrosis, and the intersections between fibroblast activity in the myocardium and the immune response in the context of HFpEF is as its infancy. Following clinical data demonstrating HFpEF patients have increased circulating neutrophil abundance, neutrophil extracellular traps (NETs) were found to contribute to cardiac fibrosis and diastolic dysfunction using a salty drinking water – unilateral nephrectomy – aldosterone (SAUNA) murine HFpEF model. Inhibition of HMGB1, a potent cardiac DAMP, reduced NET formation in the heart and ameliorated both diastolic dysfunction and fibrosis, possibly through impairing the recruitment of macrophages (99). Another major hallmark of HFpEF is metabolic syndrome, a highly pro-inflammatory condition, which particularly shapes the presentation of cardiometabolic HFpEF. In mice, the combination of advanced age, high fat diet, and intraperitoneal injection of deoxycorticosterone pivalate induces preclinical HFpEF and assembly of the NOD-like receptor protein 3 (NLRP3) inflammasome on

hyperacetylated cardiac mitochondria, driving increases in cardiac IL-1β and IL-18 abundance. Elevating cardiac concentrations of β-hydroxybutyrate (β-OHB), a dominant ketone body that is decreased in circulation in HFpEF patients, decreased mitochondrial hyperacetylation and ameliorated cardiac fibrosis and improved cardiac function (100). Lastly, preclinical HFpEF models induced by the combination of 3 hits – high fat diet to induce obesity, Nω-nitro-L-arginine methyl ester (L-NAME) to induce hypertension, and cardiomyocyte-specific deletion of the L-type Ca^{2+} channel β2a-subunit – resulted in increased cardiac $CD206^{+}CD68^{+}$ macrophages alongside increased gene expression of *Tgfb*, followed by the onset of robust perivascular and interstitial fibrosis (101). This suggests crosstalk between cardiac fibroblasts, immune cells, and cardiomoycytes in the context of metabolic and mechanical stressors induced by combined comorbidities drive HFpEF phenotypes.

While these studies are critical for elucidating how drivers of HFpEF elicit fibrotic responses, these data are largely correlative, and lack mechanistic interrogations of direct interactions between inducers of HFpEF, immune cells, and fibroblasts, as have been identified in HFrEF etiologies. Additionally, studies identifying mechanisms of immune-mediated fibrosis in specific subsets of the HFpEF patient population (e.g. cardiometabolic HFpEF or cardiorenal HFpEF) will be necessary to understand how systemic stress from HFpEF comorbidities translates to alterations in myocardial ECM composition.

Epigenetic modifications, such as the inhibition of histone deacetylases, have been shown to improve myocardial relaxation without altering blood pressure in a murine model employing uniphrectomy with deoxycorticosterone acetate-salt to induce preclinical HFpEF. In this model, while there is no detectable fibrosis by picrosirius red staining in HFpEF mice, treatment with a

clinical stage HDAC inhibitor Givinostat recovered alterations in the ECM that contributed to increased myocardial stiffness *in vivo* and blocked cardiac fibroblast activation *in vitro* (102). Additionally, mechanistic studies interrogating the potential sources of myofibroblasts in tissue have identified endothelial to mesenchymal transition (endo-MT), the process by which endothelial cells transition into a mesenchymal phenotype, as a possible mechanism of fibrosis in HFpEF. Murine studies using the SAUNA HFpEF model, which models cardiorenal HFpEF, identified endo-MT in the kidneys of HFpEF mice by the upregulation of endo-MT mRNA transcripts, namely platelet-derived growth factor receptor β (*pdgfrβ*), *snail*, fibronectin (*fn*), vimentin (*vim*), and fibroblast-specific protein 1 (*fsp1*), as well as by histology identifying $CD31^+$ cells expressing smooth muscle actin. Interestingly, the heart did not show upregulation of these transcripts, leading the authors to conclude they did not observe cardiac endo-MT at the time of analysis (103). Whether endo-MT occurs earlier or later in HFpEF or whether it occurs in specific HFpEF subsets as a consequence of particular systemic and cardiac stressors remain ongoing areas of interest. Lastly, low level transcutaneous vagus nerve stimulation (LLTS) blunted cardiac fibrosis, diastolic dysfunction, and cardiac macrophage infiltration Dahl salt-sensitive rats fed high-salt diet (104), revealing a novel intersection between the nervous, immune, and cardiovascular systems in mediating fibrotic remodeling in HFpEF.

Taken together, while cardiac fibrosis is a pathological hallmark of both HFpEF and HFrEF, fibrosis in HFpEF is somewhat phenotypically distinct from that in HFrEF. Unlike in HFrEF, where injuries occur in the myocardium that directly invoke local and recruited pro-fibrotic responses, combined comorbidities induce systemic alterations in circulating metabolic and inflammatory mediators which ultimately inflame the heart in HFpEF. Thus, rather than remodeling with focal depositions of collagen that contrast from healthy myocardium, there is a

global deposition of ECM proteins that increases the stiffness of the chamber as a whole and impacts diastolic function (Figure 3, bottom). Through preclinical studies, perhaps specific mechanisms of immune-mediated fibrosis can assist in revealing therapeutic targets in patients, or subsetting HFpEF patients by the presentation or progression of fibrosis to assist in diagnosis.

Concluding remarks

The co-existence and communication of fibroblasts and immune cells in organs through soluble factors and cellular contact reveals how these two distinct cell types participate in coordinated responses central to maintaining tissue homeostasis and rapidly promoting tissue repair after injury. Anti-inflammatory therapies are effective at treating autoimmune diseases such as RA and IBD, whereas anti-fibrotic therapies have only showed success in very specific organ conditions. In HF, no anti-inflammatory or anti-fibrotic therapies have been fully successful to date. The emerging knowledge of fibroblasts and immune cell heterogeneity and plasticity imprinted at the organ niche, where both cell types communicate and adopt functions from each other, offers the opportunity to identify potential novel therapeutic targets in organ inflammation and fibrosis. In addition, while fibrotic diseases such as HF are known to have sex-specific clinical phenotypes (105), the molecular mechanisms underlying these differences are poorly understood. While this sexual dimorphism may be attributed to ECM compositional differences or sex-dependent inflammatory mechanisms (106, 107), we propose that studying whether the intersection between fibroblast activity and inflammation is sex dependent will reveal greater mechanistic insight into the pathophysiology of HF, compared to studying these entities in isolation of one another. Despite similarities across organs, fibroblast states are also unique depending on their location in the body and in the immune response triggered when a fibrotic response needs to be put into place. Understanding these fibroblast-immune cell nodes of

communication during homeostasis and how these are modified in disease will be central to finding successful therapeutics for diseases such as heart failure, where the inflammatory and fibrotic responses co-exist.

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Figure legends

Figure 1: A. Possible fibroblast phenotypic states. Resting fibroblasts (orange) play homeostatic roles through maintaining Extracellular Matrix (ECM) proteins. They are also key producers of Colony Stimulating Factor 1 (CSF1) and Platelet Derived Growth Factors (PDGFs), as well as baseline production of interferons (IFNs) that function in homeostatic regulation. Myofibroblasts (brown) are highly proliferative and pro-inflammatory cells. They have an increased production of ECM and are largely responsible for the development of fibrosis across multiple organ systems and engage in immune cell recruitment through a variety of cytokines. Senescent fibroblasts (blue) are highly inflammatory non-dividing fibroblasts, classically characterized by an upregulation in Senesce Associated Genes (SAGs) (such as β-galactosidase/β- gal) which, when expressed, leads to the development of a Senescence Associated Secretory Phenotype (SASP). Matrifibrocytes (red) are a recently described

subpopulation of semi-senescent fibroblasts. Their exact roles in immune cell recruitment, fibrotic development, and IFN production are currently subjects of study. While all well-defined states are listed here, depending on the disease setting and cues from the microenvironment, fibroblasts may transition between several or all of these phenotypes, and others not represented herein, such as in the case of myocardial ischemia.

B. Resting fibroblasts (orange) produce pro-survival signals like CSF1 and PDGFα to macrophages and other immune cells. These same immune cells also send pro-survival signals back to the fibroblast pool. Conversely, inflamed fibroblasts (brown) produce pro-inflammatory cytokines such as CCL2, IFNγ, and IFNβ, which activate and recruit immune cells. They also self-signal through the production of Transforming Growth Factor β (TGFβ) to encourage the transformation of surrounding fibroblasts. Immune cells (right) produce pro-inflammatory cytokines such as IL-1α, IL-12, TGFβ and osteopontin to further activate the inflammatory response in fibroblasts. **C.** Fibroblasts are capable of immune cell recruitment. Resting fibroblasts (left) are activated by mechanical stresses or immune factors and begin producing pro-inflammatory and immune recruiting factors such as Monocyte Chemoattractant Protein-1 (MCP-1)/CCL2, CXCL2 and IL-11, which signal on immune cells to migrate into sites of inflammation. Additionally, fibroblasts communicate with tissue resident macrophages. These events lead to the inflamed state of the tissue. **D.** T-cells and fibroblasts interact in a bidirectional manner. T-cells can be re-stimulated by fibroblasts through fibroblast MHC II (left) and T-cells can activate fibroblasts through t cell α4 integrin binding to fibroblast VCAM-1 (right).

Figure 2: Fibroblast mediated antigen presentation plays essential functions in multiple disease contexts.

A) Rheumatoid Arthritis. Synovial neutrophils release neutrophil extracellular traps (NETs) leading to the extrusion of nuclear material along with granules meant for defense against external pathogens. NETs also contain citrullinated peptides that are internalized by human synovial fibroblasts through the RAGE-TLR endocytic pathway and IL-17B which leads to the upregulation of MHC-II. Synovial fibroblasts can then present citrullinate peptides to antigen specific CD4 T cells resulting in articular damage. **B)** Inflammatory Bowel Disease. scRNA-sec from Ulcerative Colitis patients showed expansion of a stromal population only minimally present in healthy samples that resembles gene signatures from fibroblastic reticular stromal cells, which have been shown to express MHC-II. This population was marked by increased expression of CCL19 and CCL21 (T-cell recruitment), TNFSF14/Light (T cell co-stimulation), and MHC-II invariant chain (CD74). CD74 serves as a chaperone to MHC-II and helps prevent its disassembly in the absence of bound antigen. **C)** Lung Cancer. Fibroblasts from both human tumor samples and a murine model of lung cancer express MHC-II in an IFNγ dependent manner. Fibroblast MHC-II expression leads to restricted tumor growth and increased survival by enhancing cytotoxicity and cytokine secretion of tumor infiltrating T cells. Additionally, fibroblast secreted C1q acts as a pro-survival signal for T cells within the tumor through an unknown mechanism. **D)** Heart Failure. In a model of pressure overload induced HF, infiltrating T cells can bind to CFBs through α4 integrin leading to autocrine TGFβ production and transformation into myofibroblasts. CFBs constitutively express CD80 a co-receptor for T cell activation, and Th1 cell secreted IFNγ induces expression of MHC-II allowing for cardiac peptides internalized by CFBs to be presented to infiltrating T cells promoting the progression of adverse cardiac remodeling and inflammation. This is mirrored in human patients with dilated

cardiomyopathy as those that received left ventricular assist devices designed to lessen the burden on the failing heart showed a decline in CFB HLA-DR expression by scRNA-seq.

Figure 3: Distinct presentations of cardiac fibrosis in clinical and preclinical HF with reduced vs preserved EF. (Top) Patients with ischemic or nonischemic HFrEF experience direct injury to the myocardium, resulting in a shifting of the heterogenous fibroblast pool towards a refined population in response to changes in the extracellular matrix and inflammatory mediators. This results in focal depositions of collagen which can be contrasted against the healthy myocardium. (Bottom) In contrast, in HFpEF patients, cardiac remodeling occurs in response to perturbations originating from the systemic periphery, in which combined comorbidities, such as obesity, hypertension, and age, alter circulating metabolites, immune cells, inflammatory mediators, and lipid-derived products. This results in an overall reinforcement of the ventricular ECM by additional fibrotic proteins, increasing the overall compliance of the myocardium. Thus, fibrotic remodeling in HFpEF is more global, and less local, than in HFrEF.

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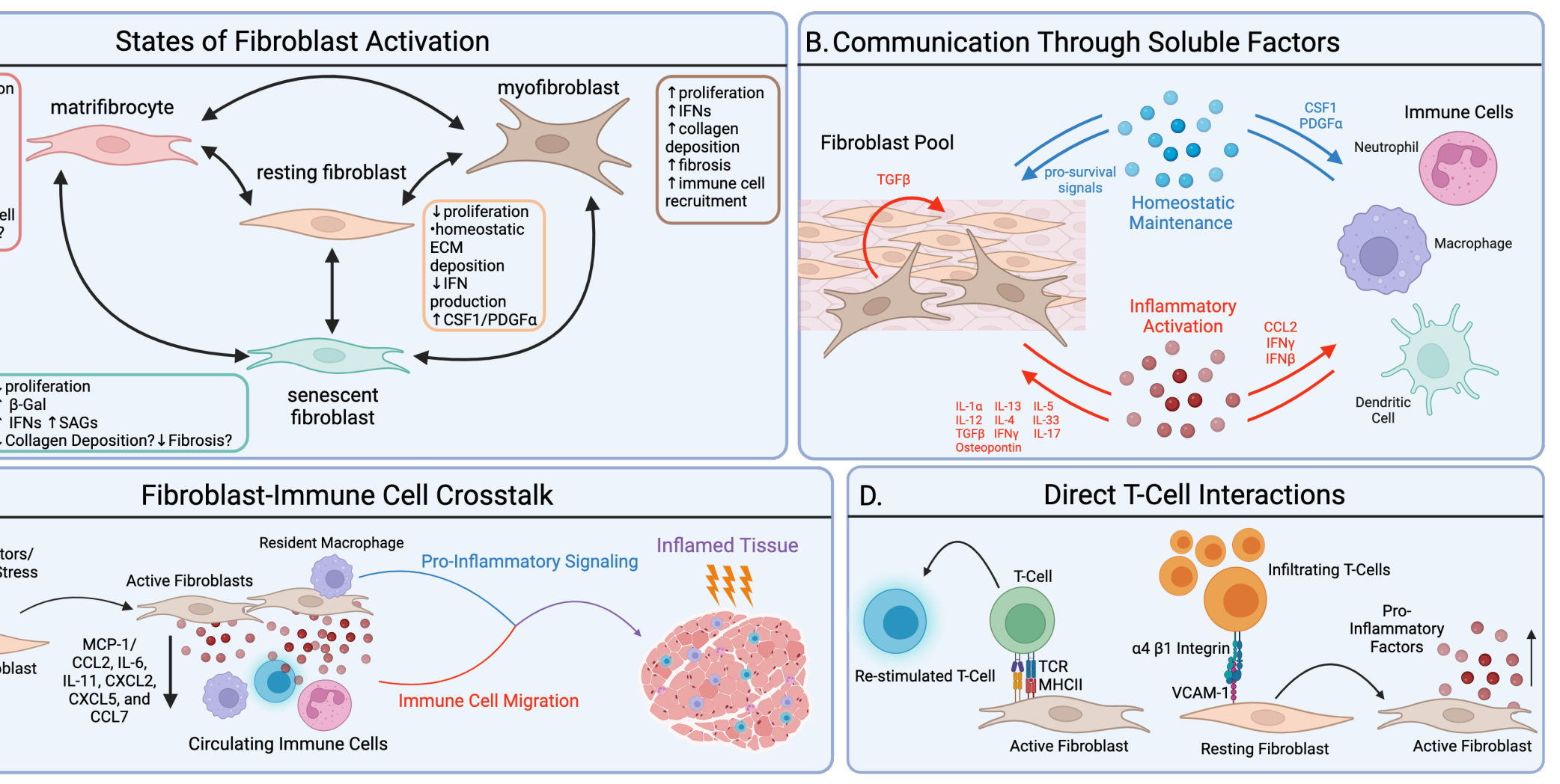
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B. Communication Through Soluble Factors
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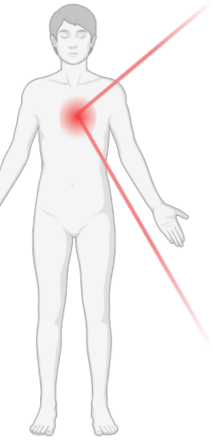
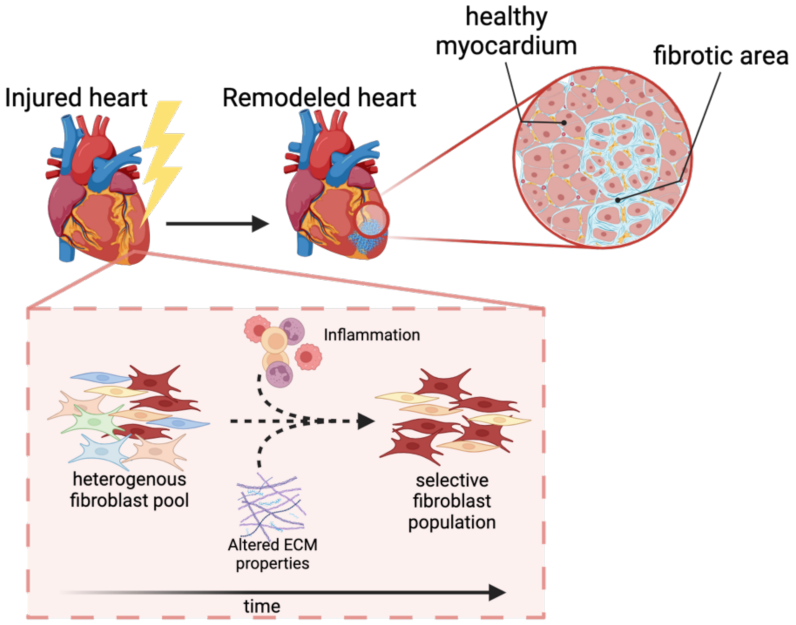
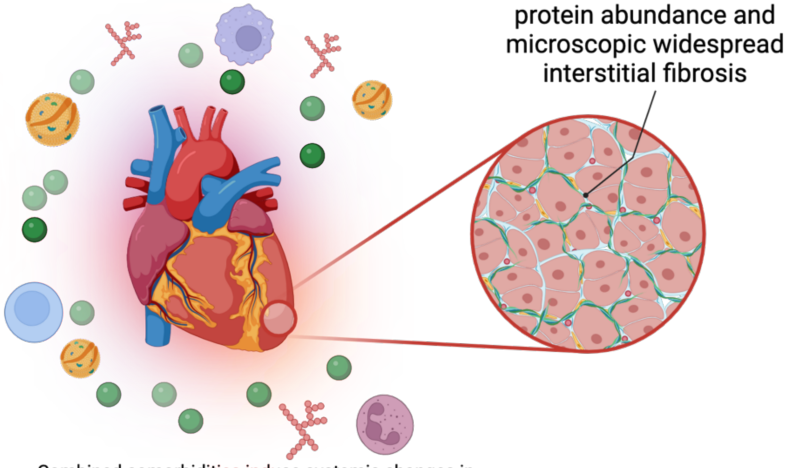


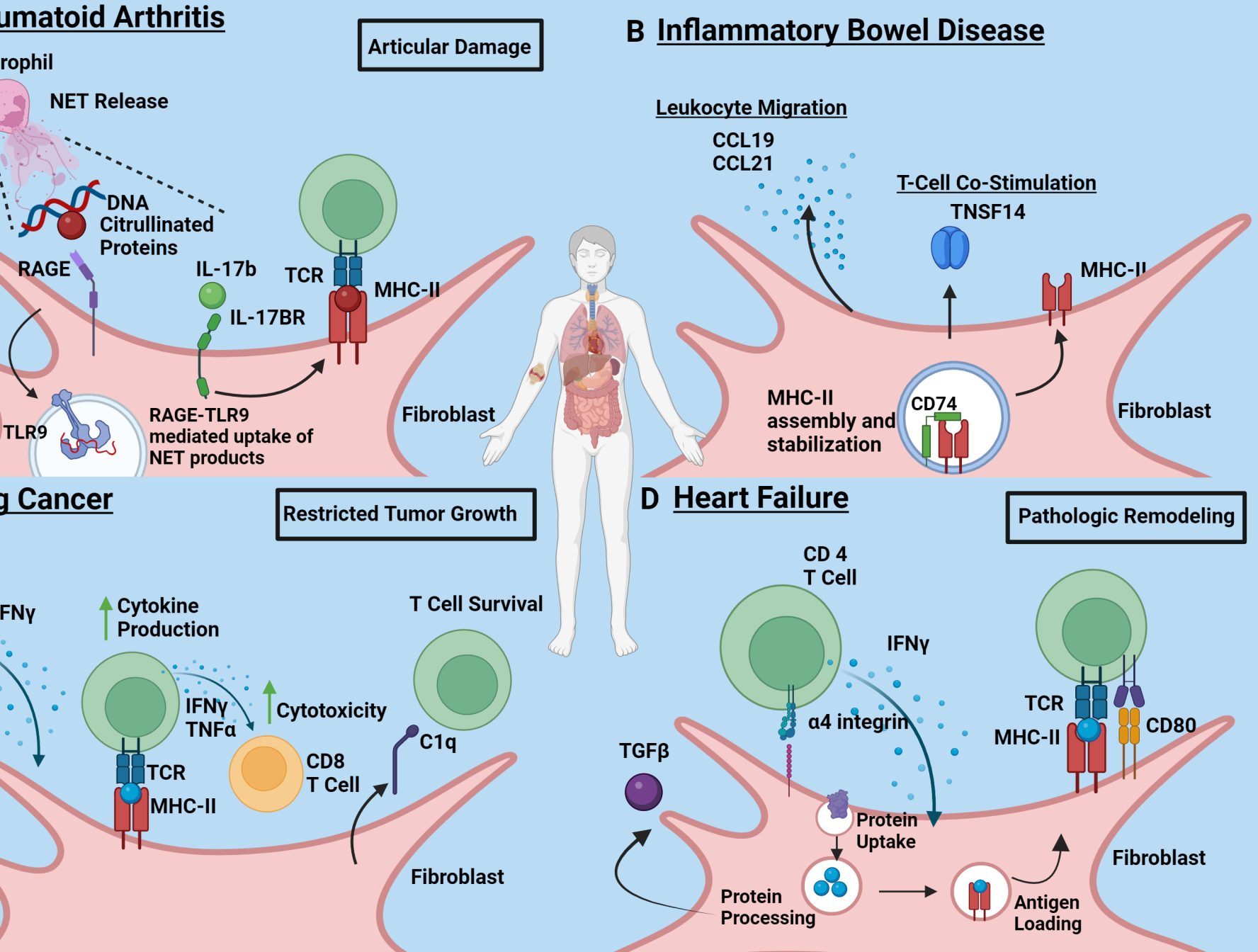


HFpEF

e, "hidden" fibrosis



Combined comorbidities induce systemic changes in lipoproteins, cytokines, immune cells, and metabolites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T-Cell Co-Stimulation
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